

13:25:16

OCA PAD AMENDMENT - PROJECT HEADER INFORMATION

04/21/93

Active

Project #: G-33-E18

Cost share #:

Rev #: 1

Center # : 10/24-6-R7660-0A0

Center shr #:

OCA file #:

Contract#: 3 R01 HL34035-05S1

Mod #: ADMIN 4/21/93

Work type : RES

Prime #:

Document : GRANT

Contract entity: GTRC

Subprojects ? : N

CFDA:

Main project #:

PE #: N/A

Project unit:

CHEMISTRY

Unit code: 02.010.136

Project director(s):

POWERS J C

CHEMISTRY

(404)894-4038

Sponsor/division names: DHHS/PHS/NIH

/ NATL INSTITUTES OF HEALTH

Sponsor/division codes: 108

/ 001

Award period: 920930 to 930630 (performance) 930930 (reports)

Sponsor amount	New this change	Total to date
Contract value	0.00	55,980.00
Funded	0.00	55,980.00
Cost sharing amount		0.00

Does subcontracting plan apply ? : N

Title: SYNTHETIC ANTITHROMBOTIC AGENTS ~~DISABILITY SUPPLEMENT R.R. PLASKON CAREER...~~

PROJECT ADMINISTRATION DATA

OCA contact: Kathleen R. Ehlinger 894-4820

Sponsor technical contact

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Security class (U,C,S,TS) : U

ONR resident rep. is ACO (Y/N): N

Defense priority rating : N

NIH supplemental sheet

Equipment title vests with: Sponsor

GIT X

Administrative comments -

ISSUED TO REVISE DELIVERABLE SCHEDULE.

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 11/01/93

Project No. G-33-E18_____

Center No. 10/24-6-R7660-0A0_

Project Director POWERS J C_____

School/Lab CHEMISTRY_____

Sponsor DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH_____

Contract/Grant No. 3 R01 HL34035-05S1_____ Contract Entity GTRC

Prime Contract No. _____

Title SYNTHETIC ANTITHROMBOTIC AGENTS DISABILITY SUPPLEMENT-R.R. PLASKON-CAREER

Effective Completion Date 930630 (Performance) 930930 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	N	_____
Final Report of Inventions and/or Subcontracts	N	_____
Government Property Inventory & Related Certificate	N	_____
Classified Material Certificate	N	_____
Release and Assignment	N	_____
Other _____	N	_____

CommentsCONTINUED BY G-33-E44._____

Subproject Under Main Project No. _____

Continues Project No. G-33-643_____

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Management	Y
Research Security Services	N
Reports Coordinator (OCA)	Y
GTRC	Y
Project File	Y
Other CARL BAXTER-FMD_____	Y
_____	N

PROGRESS REPORT SUMMARY		GRANT NUMBER HL34035-06	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR James C. Powers		PERIOD COVERED BY THIS REPORT	
APPLICANT ORGANIZATION Georgia Institute of Technology		FROM 07/01/93	THROUGH 06/30/94
TITLE OF PROJECT (Repeat title shown in item 1 on first page) Synthetic Antithrombotic Agents			
(SEE INSTRUCTIONS)			

1. Specific Aims

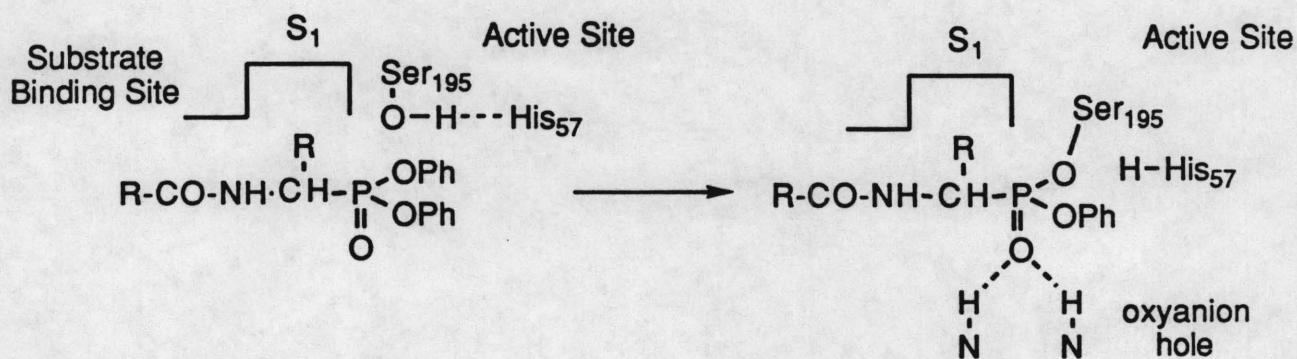
1. Design and synthesize specific peptide-related reversible transition-state inhibitors for human thrombin.
2. Design and synthesize heterocyclic irreversible mechanism-based thrombin inhibitors.
3. Utilize molecular modeling and the x-ray structure of human thrombin to improve the potency and specificity of both peptide and heterocyclic inhibitors.
4. Evaluate the inhibitory potency and specificity of all new drugs *in vitro*.
5. Evaluate the *in vivo* efficacy of the antithrombotic agents in a rabbit thrombosis model.

2. Studies and Results

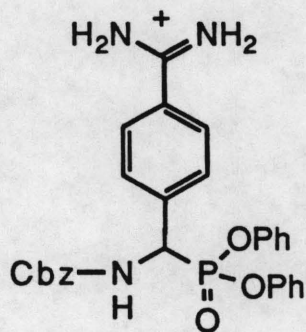
Thrombin is a key enzyme in the coagulation pathway and synthetic inhibitors for thrombin are widely considered to have significant potential for treatment of various coagulation disorders. The goal of this research is the development of effective, specific and stable low-molecular weight synthetic inhibitors for thrombin and other enzymes in the coagulation pathway that participate in the formation of thrombin.

During the past year we have focused our efforts on the synthesis of irreversible transition-state inhibitors and heterocyclic irreversible mechanism-based inhibitors for thrombin (specific aims 1 and 2). Dr. Richard Plaskon has been involved in molecular modeling studies with human thrombin (specific aim 3). The inhibitory potency of the new compounds have been evaluated *in vitro* with both synthetic substrates and in coagulation tests (specific aim 4). Since we didn't obtain potent compounds until quite recently, no animal studies were performed in the last year (specific aim 5).

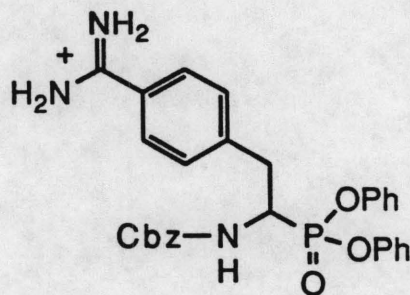
Phosphonates as Inhibitors of Thrombin. We have focused most of our synthetic effort during the last year on peptidyl derivatives of diphenyl α -aminoalkylphosphonates. These compounds have been reported previously as specific and potent irreversible inhibitors of elastases, and various chymotrypsin-like enzymes. The inhibition mechanism involves the nucleophilic substitution on phosphorus atom by the active site serine to form a tetrahedral intermediate shown below (right). This phosphonate resembles the tetrahedral intermediate formed in substrate hydrolysis. Thus, we term these phosphonates as transition-state irreversible inhibitors. The leaving group in the phosphorylation is the electronegative phenoxy group. Good interactions of the amino acid side chain of the inhibitor with the S_1 pocket and with the extended substrate binding sites (S_2 , S_3) of the enzyme are essential for effective inhibition.



We have synthesized phosphonate inhibitors containing the arginine analogs *p*-amidinophenylglycine and *p*-amidinophenylalanine, the structures are shown in the following figure. We also worked on a phosphonate arginine analog, but have not yet accomplished its synthesis. Various peptide derivatives were synthesized including analogs with the D-Phe-Pro-Arg sequence which is a potent thrombin inhibitory sequence in derivatives such as peptide chloromethyl ketones.



1, $RCO-(p-AmPhGly)P(OPh)_2$



2, $RCO-(p-AmPhe)P(OPh)_2$

Several of the inhibitors are potent irreversible inhibitors of thrombin as measure by their second order inhibition rates $k_{obs}/[I]$. The data are shown in the following table. One of the most effective derivatives is the Boc-D-Phe-Pro amidinophenylglycine derivative 5. We have also performed coagulation tests with these derivatives and find that several are effective anticoagulants (data not shown).

Table. Rates of Inhibition of Trypsin-like Serine Proteases by Peptidyl Derivatives of Diphenyl (4-AmidinophenylGly)phosphonate and (4-AmidinoPhe)phosphonate.

Inhibitor		$k_{\text{obsd}}/[I] \text{ (M}^{-1}\text{s}^{-1}\text{)}$		
		bovine trypsin	bovine thrombin	human thrombin
2	Z-(p-AmphGly) ^P (OPh) ₂	2,000	170	80
3	Z-Pro-(p-AmphGly) ^P (OPh) ₂	100	70	20
4	D-Phe-Pro-(p-AmphGly) ^P (OPh) ₂	110	730	700
5	Boc-D-Phe-Pro-(p-AmphGly) ^P (OPh) ₂	2,200	12000	11000
6	2-Np-SO ₂ -Gly-(p-AmphGly) ^P (OPh) ₂	470	170	
8	Z-(p-AmPhe) ^P (OPh) ₂	24		0.2
9	2HCl·(p-AmPhe) ^P (OPh) ₂	32		2.2
10	Z-Pro-(p-AmPhe) ^P (OEt) ₂	NI		NI
11	Boc-D-Phe-Pro-(p-AmPhe) ^P (OPh) ₂	130		0.7
12	D-Phe-Pro-(p-AmPhe) ^P (OPh) ₂	50		54
	Ac-D-Phe-Pro-Arg ^P (OPh) ₂			1200

3. Significance

Thrombin has a central regulatory role in hemostasis and is formed by both the intrinsic and extrinsic pathways of blood coagulation. The major function of this serine protease is the cleavage of fibrinogen to form fibrin clots, but thrombin also activates factor V, VIII, XIII and protein C which are important in the control of hemostasis and thrombosis. In addition, thrombin can stimulate platelet secretion and aggregation in blood, and mediate other nonhemostatic cellular events.

Thrombin is a well recognized target for the design of new antithrombotic agents since this serine protease is such a powerful trigger in thrombus formation in the blood. Considerable effort is being devoted to the development of new antithrombotic agents since the currently available drugs such as heparin often cause bleeding when used therapeutically and are unable to prevent the occlusive complications in atherosclerotic vascular disease or reocclusion following successful thrombolysis. Although much of this activity has been focused on high molecular weight thrombin inhibitors such as hirudin or low molecular weight platelet aggregation inhibitors, increasing research is being devoted to the development of suitable small synthetic thrombin inhibitors. In the future, it is likely that synthetic thrombin inhibitors will supplement or even replace currently used antithrombotic agents in a variety of therapeutic situations.

The phosphonate inhibitors which we have developed in this research have the advantage to being stable in plasma, highly specific for their target protease and fairly potent. We believe they have considerable potential for development as antithrombotic agents.

4. Plans

Design and Synthesis. We plan to continue to improve the amidinophenylglycine and amidinophenylalanine derivatives as thrombin inhibitors and to extend this class of inhibitors to other enzymes particularly factor VIIa and factor Xa. Dr. Plaskon has begun modeling studies with the phosphonates and we expect to be able to use his results to direct our design and synthesis work. Thus far it appears that the phosphonate are binding in an expect and unique manner to thrombin which should allow substantial improvement in their inhibition rates through structural modifications.

Vertebrate Animal Studies. Since no potent inhibitors were synthesized until recently, no animal work was carried out during the second year of this research. We plan to carry out preliminary animal studies shortly (May 1993) with three of the inhibitor molecules shown in Table I.

Publications

Synthetic Substrates And Inhibitors For Serine Proteases From Lymphocytes, Mast Cells, Semen, and Blood, Powers, J. C., Odake, S., Ueda, T., Hudig, D., Graves, H., Kamarei, M., and Kam, C.-M. (1992) in *Recent Progress on Kinins. Part I. Pharmacological and Clinical Aspects of the Kallikrein-Kinin System. Proceeding of the Conference "Kinin 1991 Munich", Agents and Actions Supplements 38/II* (Bönner, G., Fritz, H., Unger, T., Roscher, A., and Luppertz, K., eds), pp 128-135, Birkhauser Verlag, Basel.

Synthetic Substrates and Inhibitors of Thrombin, Powers, J. C., and Kam, C.-M. (1992) in *Thrombin: Structure and Function* (Berliner, L. J., Ed.) pp 117-158, Plenum Publishing Corp., New York.

Inhibition of Porcine Pancreatic Elastase by 7-Substituted 4-Chloro-3-Ethoxyisocoumarins: Structural Characteristics of Modeled Noncovalent Complexes Relate to the Measured Kinetics, Plaskon, R. R., Kam, C.-M., Kerrigan, J. E., Burgess, E. M., Powers, J. C., and Suddath, F. L. (1993), *Arch. Biochem. Biophys.* 300, 588-597.

Michaelis Complexes of Porcine Pancreatic Elastase with 7-[alkylcarbamoyl]amino]-4-Chloro-3-Ethoxyisocoumarins: Translational Sampling of Inhibitor Position and Kinetic Measurements, Plaskon, R. R., Kam, C.-M., Burgess, E. M., Powers, J. C., and Suddath, F. L. (1992) *Proteins* 13, 141-151.

Substituted Isocoumarins As Inhibitors of Complement Serine Proteases, Kam, C.-M., Oglesby, T. J., Pangburn, M. K., Volanakis, J. E., and Powers, J. C. (1992), *J. Immunology* 149, 163-168.

**PROGRESS REPORT-DR. RICHARD R. PLASKON
RESEARCH SUPPLEMENT TO PROMOTE THE RECRUITMENT OF INDIVIDUALS
WITH DISABILITIES INTO BIOMEDICAL RESEARCH CAREERS**

A major portion of the research conducted by Dr. Plaskon during the first year of this supplement involved the modeling of isocoumarin mechanism-based thrombin inhibitors into the active site of human α -thrombin. The compounds modeled include the most potent derivatives of 4-chloro-3-isothiureidoalkoxyisocoumarin inhibitors of human α -thrombin with various 7-amino substituents. A related isocoumarin thrombin inhibitor has previously been shown to be an anticoagulant in a rabbit model of coagulation. Dr. Plaskon's results are included in a manuscript which will shortly be submitted to the J. Medicinal Chemistry.

To accomplish the energy minimizations required for the molecular modeling, semiempirical quantum mechanical calculations were successfully performed on portions of the human α -thrombin active site to obtain the partial atomic charges. Results of the modeling revealed that H-bonding between the Lys-60F NH_3^+ of thrombin and the polar portion of the isocoumarin's 7-substituent was related to the inhibitor's potency. The potency of one inhibitor was correctly predicted from the calculation, but its potency was no greater than that of the other isocoumarin inhibitors modeled. From the modeling, Dr. Plaskon predict that an isocoumarin with a particular substituent would be expect to have greater potency. But thus far, we have been unable to isolate this isocoumarin for testing.

A "leucine pocket" in the human α -thrombin active site was discovered while viewing the modeled structures. Based on the location of the pocket relative to that of the irreversible inhibitor D-Phe-Pro-Arg-chloromethylketone (FPRCK) in the FPRCK—human α -thrombin complex crystal structure, it is thought that it might correspond to a S' site (i.e., a site where a side chain of an amino acid on the carbonyl side of the scissile bond interacts). In the second year of the supplement, Dr. Plaskon will determine what groups can interact with this pocket. It is expected that long unbranched hydrocarbon chains would insert into the pocket. An isoleucine side chain might insert as well.

To model inhibitors into the active site of thrombin, initial *in vacuo* inhibitor structures were obtained from semiempirical quantum mechanical calculations. These structures revealed that the orientation of the phenyl group of isocoumarin inhibitors which contain phenyl groups in their 7-substituent can be correlated with inhibitor potency. This result, combined with their modeling into the human α -thrombin active site, suggests that the phenyl group alters the relative orientation between the 7-substituent's polar region and the Lys-60F NH_3^+ of thrombin. Thus Dr. Plaskon is able to predict the inhibitory potency of new synthetic isocoumarin inhibitors before the compounds are synthesized. This results in a considerable savings in time and expense since only structures which have the greatest probability of being potent inhibitors will be synthesized.

Dr. Plaskon has begun to model the binding of phosphonate inhibitors, in particular peptidyl derivatives of diphenyl (4-amidinophenylGly)- and (4-amidinoPhe)phosphonates, to the human α -thrombin active site. Semiempirical quantum mechanical calculations have been used to generate *in vacuo* structures of benzyloxycarbonyl derivatives of diphenyl (4-amidinophenylGly)- and (4-amidinoPhe)phosphonates. At present, only the benzyloxycarbonyl derivative of the diphenyl (4-amidinoPhe)phosphonate has been docked in thrombin's active site. A comparison of this docked structure with the X-ray crystal structure of D-Phe-Pro-Arg-chloromethylketone complexed with human α -thrombin was made. The D-Phe-Pro-derivative of diphenyl (4-amidinoPhe)phosphonate can't bind to thrombin in an orientation similar to that of the chloromethyl ketone without altering the orientation of the phosphonate so that it will not easily react with the active site serine. This would explain the phosphonate's low inhibitory potency. Additional modeling studies will be conducted in the second year of the supplement to help determine if this explanation is correct. Such studies should also reveal changes that could be made in the peptidyl groups which would result in phosphonate inhibitors of greater potency.

Publications

Inhibition of Porcine Pancreatic Elastase by 7-Substituted 4-Chloro-3-Ethoxyisocoumarins: Structural Characteristics of Modeled Noncovalent Complexes Relate to the Measured Kinetics, Plaskon, R. R., Kam, C-M., Kerrigan, J. E., Burgess, E. M., Powers, J. C., and Suddath, F. L. (1993), Arch. Biochem. Biophys. 300, 588-597.

Michaelis Complexes of Porcine Pancreatic Elastase with 7-[alkylcarbamoyl]amino]-4-Chloro-3-Ethoxyisocoumarins: Translational Sampling of Inhibitor Position and Kinetic Measurements, Plaskon, R. R., Kam, C-M., Burgess, E. M., Powers, J. C., and Suddath, F. L. (1992) *Proteins* 13, 141-151.